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14. ABSTRACT

Accomplishments during reporting Year 3. (1) We have conducted single-cell electrophysiological examination of transmission after chronic contusion SCI and obtained results demonstrating rewiring of corticospinal projections to individual interneurons and motoneurons. (2) The recombinant single chain (scFv) antibody has been created (patent pending), transferred to PENN vector core, where the new vector, i.e. AAV10 encoding the anti-NG2 scFv has been constructed. (3) Determined the optimal AAV vector serotype for NG2-Ab delivery: we examined expression pattern of six AAV-gfp serotypes (AV1,2,5,9,10,11) in damaged spinal cord; AAV10-gfp induced best transduction of spinal cord tissue following contusion SCI. (4) Examined AAV10-mediated delivery of NG2-Ab combined with neurotrophin NT-3 following SCI in rats and found significant recovery of function in treated vs control groups. Thus all specific aims are accomplished. Moreover, our study proved AAV10-NG2-Ab construct as novel effective and clinically relevant treatment to facilitate recovery after SCI. We apply for 6-moths no-cost extension to conduct immunochemistry analyses of spinal cord tissue from this completed experiment.

15. SUBJECT TERMS Completed: (2) proved that acute injections of anti-NG2 antibodies (Ab) prevent block of axonal conduction induced by acute NG2. (3) chronic delivery of NG2-Ab via mini-pump improves axonal conduction, plasticity and recovery of function after chronic SCI. Successfully created scFv Ab; its functional effects will be examined at Year3 to complete Project.

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Introduction

Regeneration of fibers in damaged spinal cord is quite limited and strategies to strengthen surviving connections in partially injured spinal cord appear to be more feasible for improving recovery of function (Arvanian et al. 2006a; Alilain et al., 2011; Garcia-Alias et al. 2011; Schnell et al. 2011). After SCI the diminished ability of the spared fibers to transmit signals during chronic stage of contusion (Hains et al. 2004; Arvanian et al. 2006b; James et al. 2011), compression (Nashmi and Fehlings 2001; Ouyang et al. 2010) and lateral hemisection (HX) (Arvanian et al. 2009) injuries have been reported. We have developed a method of intra-axonal recordings from anesthetized adult rats. Using this method we found that transmission deficits in damaged spinal cord after chronic HX are the result of reduced conduction in uncut axons (Hunanyan et al. 2011). Since elevated level of chondroitin sulfate proteoglycans (CSPGs) in the vicinity of the injury has been reported to be a major obstacle for recovery after SCI (Snow et al., 1990; Jones et al., 2002), the general strategy of this proposal is: (i) to identify individual CSPGs that might be responsible for the conduction deficits in the surviving fibers, (ii) to design a specific treatment that will neutralize these CSPGs, and (iii) to determine whether neutralization of these factors will strengthen synaptic effects of surviving descending fibers and improve functional recovery in adult rats after HX injury. Keeping up with the schedule of our study, During Year1 we found that among CSPGs which level is up-regulated in the vicinity of SCI, NG2 is blocks axonal conduction, but other CSPGs do not. During Year2 of the Project we have completed research tasks for Year 2 and examined effects of NG2-Antibody delivered acutely and via osmotic mini-pump following lateral hemisection (HX) SCI, as described in our SOW. During year 3 we have completed most of research tasks for year 3. We have examined transmission in damaged spinal cord following contusion SCI in adult rats, which is more realistic model of spinal injuries. Moreover, during year 3 we have successfully created the recombinant single chain (scFv) antibody (patent is pending). The plasmid has been transferred to PENN vector core, where the AAV10 viral vector encoding the anti-NG2 scFv has been constructed. The choice of AAV-10 serotype for this new construct was based on our experiments that revealed that among several AAV-gfp serotypes that we tested (AAV1,2,5,9,10,11), AAV10-gfp induced best transduction of spinal cord tissue following contusion SCI. We further examined AAV10-mediated delivery of NG2-Ab in rats that received either hemisection or contusion SCI and intraspinal injections of AAV10-NG2-Ab. We found that rats that received SCI and AAV10-NG2-Ab treatment exhibited significantly better recovery of locomotor function compared with control group that received identical SCI and control AAV10-gfp injections. Thus all specific aims are accomplished. Moreover, our study proved that AAV10-NG2-Ab construct that we created may be a novel, effective and clinically relevant treatment to facilitate recovery after SCI. During Year 2013 results of experiments supported by DOD Proposal has been reported in a form of three invited talks at international symposiums (Step-by-Step Symposium Barcelona Spain, European Neuroscience Forum Prague and Working-to-Walk Symposium Boston), and one review and two articles have been published in scientific journals. Overall the DOD-funded Project is successful and our one paper has been chosen for "Best Research paper" award (European Neuroscience Forum, Prague 9/10/2013).

We apply for 6-moths no-cost extension to complete post-mortem immunochemistry analyses in order to determine the nature of cells that were transduced by AAV10-NG2-Ab. As a result of the extensive requirements and overall process required for the new hires to work within the Northport VA Medical Center, some of our research was delayed by several months. Because of this delay, we have some immunochemistry analyses that we would need to finish and submit another three papers for publication. The no-cost 6-month extension of the contract will ensure successful completion of our study.

Body

1. Specific Aim #1 of our project has been accomplished during Year 1. We have examined and compared acute effects of several individual CSPGs on axonal conduction and synaptic transmission in intact spinal cord. We found that among CSPGs whose levels are mostly elevated after spinal cord injuries (i.e. NG2, neurocan and aggrecan), NG2 induced a dramatic depression of axonal conduction and transmission to individual motoneurons in intact spinal cord, but neurocan or aggrecan did not alter axonal conduction. Results of these experiment have been published (Hunanyan et al., 2010).

2. Specific Aim #2 has been accomplished during Year 2. We have examined acute effects of anti-NG2-antibody (Ab) (made in Dr. Levine lab). Acute experiments revealed that acute intraspinal injections of NG2-Ab prevented an acute block of conduction induced by NG2. These results suggest the possibility that intraspinal injections of the NG2-Ab may be useful for local neutralization of NG2 in the areas where needed such as the site of SCI. Results of these experiment have been published (Petrosyan et al., 2013).

3. Specific Aims #3,4. A portion of SA#3 and SA#4 related to chronic administration of NG2-Ab via osmotic mini-pump after chronic SCI has been accomplished during Year2. We found that chronic delivery of NG2-Ab via osmotic mini-pump improves recovery of locomotor function, synaptic transmission and anatomical

plasticity after a lateral hemisection (HX) SCI. Results of these experiments have been published (Petrosyan et al., 2013).

Moreover, during past year (2013) we have successfully developed new AAV-based viral vector expressing NG2-antibody and successfully examined ability of this new construct to improve recovery of motor function following hemisection SCI, as well as contusion, which is more realistic SCI model. Below we describe the experiments that have been conducted and the results of these experiments.

(1) First single-cell electrophysiological examination of transmission after chronic contusion SCI.
Contusion SCI in adult rat has been widely accepted as realistic SCI model. Transmission through descending pathways to lumbar motoneurons, although important for voluntary walking in humans and rats, has not been fully understood at the cellular level in contusion models. Major descending pathways innervating lumbar motoneurons include those at corticospinal tract and ventrolateral funiculus (VLF). We examined transmission and plasticity at synaptic pathways from dorsal corticospinal tract (dCST) and VLF to individual motoneurons located in ventral horn and interneurons located in dorsomedial grey matter at lumbar segments following thoracic chronic contusion in adult anesthetized rats. To accomplish this we used intracellular electrophysiological recordings and performed acute focal spinal lesions during the recordings.

Methods.

Spinal cord injury. All procedures were performed on adult, female Sprague-Dawley rats (~210 g) in compliance with Institutional Animal Care and Use Committee policies at SUNY-Stony Brook and the Northport VAMC. Animals were anesthetized with 3% isoflurane in 100% O2 in an induction chamber and then transferred to a facemask delivering 1.5% isoflurane in 100% O2 to maintain anesthesia during surgery. Petrolatum ophthalmic ointment (Dechra Veterinary Products) was applied to the eyes to prevent desiccation. Rats were placed on a water circulated heating pad to maintain body temperature at 36.5-37^{0}C. Before surgery, animals received a subcutaneous injection of Buprenorphine (0.01mg/kg) to reduce post-operative pain. Contusion injury was performed at T10 spinal level using computer controlled IH-0400 Impactor device (Precision System and Instrumentation). Briefly, a dorsal laminectomy was performed to expose T10 spinal cord. Vertebral bodies were fixed at T8 and T10 using Adson forceps, and 150 kdyn force was used to induce a moderate severity contusion injury. The actual mean impaction force was 154 ± 1 kdyn with tissue mean displacement of 1021± 63µm. After the injuries, the muscles were sutured with 4-0 monocryl (Ethicon) and skin was closed with wound clips followed by subcutaneous injections of antibiotic (Baytril, 5 mg/kg) and 5 ml sterile-lactated Ringer`s solution. Injections of antibiotic, analgesic, and Ringer`s solution were administered for 3 days post-injury. Histological analyses of the injury epicenter revealed complete disruption of the dCST in all SCI animals, 9-11 weeks following thoracic contusion (Fig. 1a).

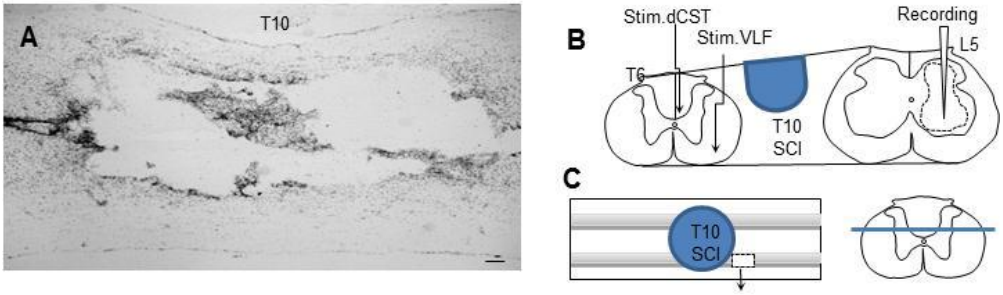

Figure 1. *Corticospinal tract damage induced by thoracic contusion. (**A**) Horizontal section at the site of injury (at T10), taken at the level of the dCST 10 weeks after 150 kdyn contusion showing complete disruption of the dCST. (**B**) Diagram showing the positions of the stimulating electrodes in dCST and VLF at T6 and intracellular recording electrode at L5 in relation to contusion.*

Recording procedure. All electrophysiological recordings were performed 9-11 weeks post-injury. Rats were deeply anesthetized with a ketamine (80 mg/kg, 0.5 ml)-xylazine (10 mg/kg, 0.5 ml) mixture (intraperitoneal injections) followed by 1/5 of initial dose administered intramuscularly throughout experiment when needed. Tracheotomy was performed to provide artificial ventilation if necessary. Heart rate and expired CO2 were monitored continuously and body temperature was maintained at 36-37°C using an automated controlled heating pad. Two dorsal laminectomies of the spinal cord were performed to expose T6–T8 (for placement of the stimulation electrodes and for acute dorsal column or lateral hemisection lesions) and L1–L6 (for placement of the recording electrode). L1-L6 ventral spinal segments were fixed tightly between custom-made bars to prevent movement of spinal cord during recordings. Intracellular (Axoclamp 900A amplifier,

Molecular Device) recordings were performed from L5 spinal segment using a sharp glass microelectrode (50-80 MΩ resistance, filled by 3M K-acetate) attached to a hydraulic microdrive (David Kopf Instruments), which precisely (with accuracy to 1 μm) measures the depth of the tip of the electrode. The glass microelectrode was positioned perpendicular to the cord, between the midline and the dorsal root entry zone and recordings were performed starting from dorsal surface. We recorded from interneurons located in dorsomedial grey matter (at depth 0.05-1.2 mm) and ventral horn motoneurons (at depth 1.3-2.3 mm) in each rat. All neurons were identified by their ability to generate action potentials in response to depolarizing current injection through the same recording electrode. Motoneurons were identified by their antidromic action potential to electrical stimulation of cut L5 ventral root. The resting membrane potential of neurons used for analysis ranged from -55 to - 65 mV. Recordings were collected from 10-15 neurons in each rat. Maximum responses from each neuron (10-30 consecutive responses/cell) were averaged. These average values were compared over all animals and for statistical analysis we used both the number of animals and the total number of cells in each group.

Electrical stimulation protocols. We examined synaptic responses of these neurons evoked by electric stimulation (70 μs duration, 1 Hz frequency; using A300 Pulsemaster/A360 Stimulus Isolator, World Precision Instruments) of dCST and VLF, at T6. For electric stimulation of dCST and VLF we used two identical tungsten electrodes inserted into spinal cord at the appropriate depth so that the positions were not changed throughout the experiment. In all experiments, electric stimulations were applied ipsilateral to recording. For stimulation of VLF, a tungsten electrode (resistance: 300 KΩ; FHC, Bowdoin, ME) was positioned between the dorsal root entry zone (at an angle of ~200, tip directed caudally) and the lateral edge of the cord and lowered to the depth ~1.7 mm (Arvanian et al. 2009). For electric stimulation of dCST a second tungsten electrode was positioned 0.1mm from dorsal midline (at an angle ~150, tip directed caudally) and lowered to the depth ~ 1 mm (Hunanyan et al. 2012). The position of stimulation and recording electrodes are schematically presented in Fig. 1b.

Acute dorsal column lesion and lateral hemisection during intracellular recordings. To examine synaptic pathways through dCST and VLF, we performed an acute focal dorsal column lesion followed by a lateral hemisection at T8, i.e. between stimulating and recording electrodes, ipsilateral to the recording/ stimulation side respectively (see Fig. 1B). We performed an acute lesion of the dorsal column to interrupt the transmission through dorsal corticospinal fibers and an acute lateral hemisection to interrupt transmission through lateral white matter (described in Schnell et al. 2011). Each acute lesion was performed after recording from several neurons in the same rat. During continuous recording from the "last" neuron (either a ventral horn motoneuron in some animals or a dorsomedial interneuron in other animals) we performed the acute lesions and examined effect of the acute lesion on the responses evoked by stimulation of both dCST and VLF. Synaptic responses from both dCST and VLF were then measured from several additional interneurons and motoneurons. Note that after dorsal column or lateral hemisection lesions the cells were able to generate action potentials evoked by depolarizing current through the recording electrode (data not shown), thus suggesting that after acute lesions the recorded neurons remained viable and the loss of the excitatory postsynaptic potentials (EPSPs) was the result of interruption of the transmission pathway.

Results.

Non-injured spinal cord.

Motoneurons: receive many monosynaptic projections from VLF and few polysynaptic projections from dCST. Consistent with our previous study in adult non-injured rats (Arvanian et al. 2009), electric stimulation of VLF evoked monosynaptic excitatory postsynaptic potential (EPSP) in almost all recorded L5 ventral horn motoneurons. Stimulation of dCST, however, evoked responses only in about 44% of motoneurons recorded. A noticeable difference between responses evoked by stimulation of VLF and dCST in the same motoneurons was that all VLF-evoked responses were monosynaptic (Fig. 2A/traces 1a), while dCST-evoked responses were polysynaptic (Fig. 2A/traces 2a). Evidence that motoneurons responses evoked by VLF are most probably monosynaptic is based on short latency, steep rising phase and negligible fluctuation in both latency and amplitude of these responses (amplitude 5 ± 0.5 mV; latency 1.4 ± 0.19 ms; n = 7 rats, 73 cells) (Fig. 2A/traces 1a) as previously discussed (Arvanian et al. 2009). The following results suggest that motoneuron responses from dCST are most probably polysynaptic: (a) these responses had smaller amplitude and longer latency (amplitude 2.9 ± 0.3 mV; latency 3.6 ± 0.4 ms; n = 7 rats, 57 cells; $p < 0.05$; Fig. 2A/traces 2a) compared to VLF-evoked EPSPs in the same motoneurons; (b) EPSPs evoked in the motoneurons following electric stimulation of dCST displayed a less steep rising phase and were variable in shape and peak deflection, compared to abrupt VLF-evoked responses in same motoneuron. The major difference between dCST- and VLF-evoked motoneurons responses was their sensitivity to acute lesions of the dorsal column. Acute transection of the dorsal column, between recording and stimulating electrodes, resulted in elimination of dCST-evoked responses in motoneurons (Fig. 2A/traces 2b), but VLF-evoked responses of the same

motoneuron sustained (Fig. 2A/traces 1b). The VLF-evoked responses in motoneurons that sustained following dorsal column lesion were abolished after further ipsilateral hemisection (not shown). These results strongly suggest that in non-injured spinal cord the same motoneurons in lumbar ventral horn may receive monosynaptic inputs from VLF and polysynaptic inputs from dCST; moreover, motoneurons responses from these two inputs are realized through activation of two independent synaptic pathways.

Interneurons: do not receive functional projections from VLF, but receive both monosynaptic and polysynaptic projections from dCST. Electric stimulation of VLF (that evoked monosynaptic responses in ventral horn motoneurons, Fig. 2A/traces 1a), did not induce measurable synaptic responses in dorsomedial interneurons in the same animal (Fig. 2A/traces 3a). Stimulation of dCST, however, evoked either monosynaptic (shorter latency of 1.6 ± 0.2 ms, about 30% of cells recorded) or polysynaptic (longer latency of 3.1 ± 0.3 ms, about 70% cells) EPSPs (amplitude 2.5 ± 0.4 mV, Fig. 2A/traces 4a, n = 7 rats, 57 cells) in these interneurons. After acute lesion of the dorsal column all (monosynaptic and polysynaptic) EPSPs of dorsomedial interneurons evoked from dCST were completely abolished in these non-injured animals (Fig. 2A/traces 4b). These results suggest that in non-injured spinal cord dCST has functional synaptic connections with dorsomedial lumbar interneurons, while VLF does not.

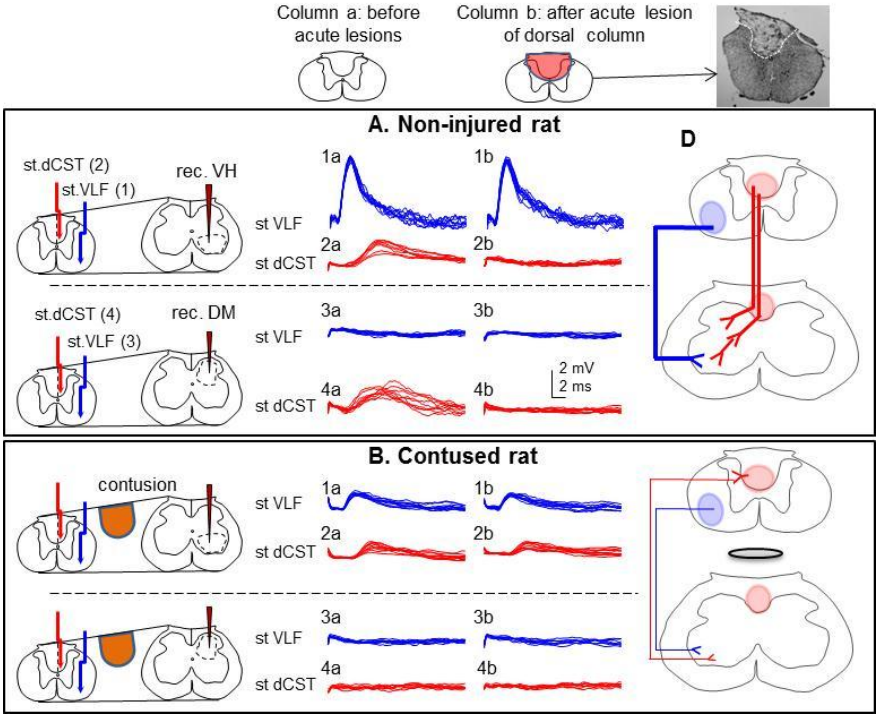

Figure 2. *Rearrangement of synaptic circuits after chronic thoracic contusion. Consecutive traces of EPSPs evoked in L5 ventral horn (VH) motoneurons and dorsomedial (DM) interneurons from T6 ipsilateral dCST (red traces) and VLF (blue traces).*
(A) Non-injured rat. Traces: responses recorded before (1a-4a) and after (1b-4b) acute lesions of the dorsal column at T8, i.e. between stimulation and recording electrodes.
(B) Contusion SCI rat.
(D) Diagrams illustrate the possible synaptic projections.

Perturbation of synaptic projections following chronic thoracic contusion

Motoneurons: projections from VLF sustained, but weakened; projections from dCST through dorsal column abolished, but new weak synaptic connections from dCST through ventrolateral white matter to lumbar motoneurons were formed spontaneously. After chronic thoracic contusion injury, stimulation of VLF evoked weak but still measurable EPSPs in ventral horn motoneurons (1.4 ± 0.18 mV, n = 6 rats, 47 cells) and longer latency (2.4 ± 0.2 ms) compared with uninjured animals (Fig. 2B/traces 1a). Acute lesion of the dorsal column at T8 did not alter these responses (Fig. 2B/traces 1b). These results suggest that after contusion SCI projections from VLF to ventral horn motoneurons sustained, although these responses were dramatically attenuated. Stimulation of dCST evoked small amplitude (1.2 ± 0.09 mV) polysynaptic EPSP responses in motoneurons and these responses appeared to be de-novo (see Fig. 2B/traces 2a; Table 1). A striking result was that in contused spinal cords these dCST-evoked responses sustained after acute lesions of T8 dorsal column (Fig. 2B/traces 2b); these responses were abolished after further ipsilateral hemisection (not shown). Note that corresponding dCST-evoked responses in uninjured cord were abolished after similar acute lesion of the dorsal column (Fig. 2A/traces 2b). These results strongly suggest that after chronic mid-thoracic contusion injury dCST fibers spontaneously make a small number of new functional synaptic connections around the contusion cavity, through spared ventrolateral white matter, to lumbar L5 motoneurons.

Interneurons: still do not receive projections from VLF and lost all projections from dCST. Recordings from dorsomedial interneurons revealed that responses from VLF (that were not evident in non-injured rats, Fig.2A/traces 3a) were still lacking in contused animals (Fig. 2B; traces 3a). Functional synaptic inputs from dCST to interneurons (that were present in non-injured rats, Fig. 2A/traces 4a), were completely abolished in

contused rats (Fig. 2B/traces 4a). These results demonstrate that dorsomedial interneurons completely lost all functional projections from dCST after contusion SCI.

Conclusions.
We directly demonstrate that after thoracic T10 chronic contusion the disrupted dCST axons spontaneously form new synaptic contacts with individual motoneurons, extending around the contusion cavity, through spared ventrolateral white matter. These detour synaptic connections are very weak and strengthening these connections in order to improve function may be a target for therapeutic interventions following SCI.

(2) **Novel AAV vector-based construct expressing NG2-antibody has been created**.
Our studies during year 2 of the Project revealed that intrathecal infusion of NG2-Ab for 2 weeks, via osmotic minipump, partially improved the following deficits induced by chronic mid-thoracic lateral hemisection (HX) injury: (i) synaptic transmission to lumbar motoneurons; (ii) retrograde transport of Fluororuby (FR) anatomical tracer from L5 to L1, (iii) density of 5-HT-positive fibers and (iv) recovery of motor function after lateral hemisection SCI . (Petrosyan et al., 2013 publication describing these effects of purified NG2-Ab administered via mini-pump following a HX SCI is attached). A downside of administration of NG2-antibody vial mini-pump in clinics is that it requires intrathecal implantation of the catheter, which can be potentially clogged and thus limit prolonged administration of NG2-Ab.

Therefore, we have recently successfully developed new tools for clinically-relevant prolonged delivery of NG2-Ab, i.e. gene transfer of anti-NG2 monoclonal antibody using adeno-associated vector (serotype 10; AAV10; collaboration with Dr. Levine and PENN vector core; patent is pending). The cDNA construct for NG2-Ab has been created (Levine lab), consisting of a signal peptide, heavy chain variable region, linker region consisting of serines and glycines, light chain variable region, and a 6 histidine tag. The cDNA was then inserted into a plasmid by Integrated DNA technologies. HEK293 cells were transfected and expression of NG2-Ab has been confirmed. cDNA for NG2-Ab and the plasmid, were then sent to the PENN vector core, where they were successfully inserted into AAV-10 viral vector.

(3) **Determined optimal AAV vector serotype for NG2-Ab delivery**.
Our choice of AAV-10 serotype AAV-mediated delivery of NG2-Ab was based on our study in which we have examined the expression pattern of six AAV-gfp serotypes (AV1,2,5,9,10,11) in damaged spinal cord. We found that AAV10-gfp induced best transduction of spinal cord tissue following contusion SCI.

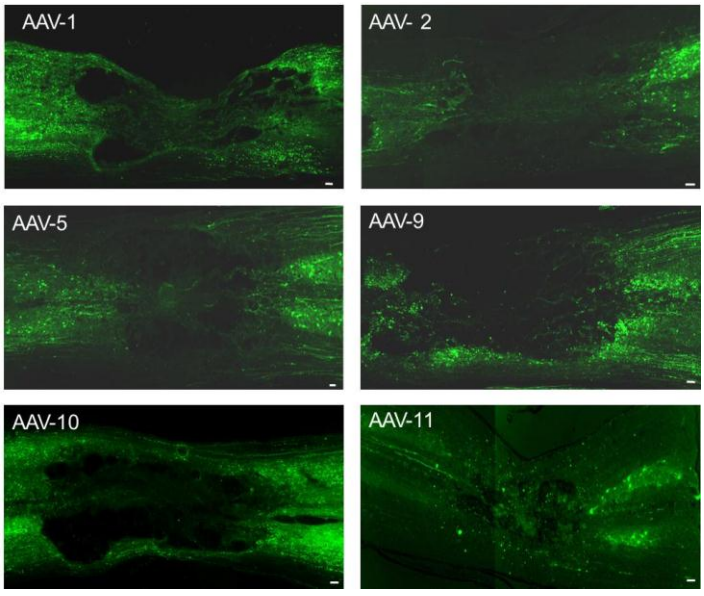

Figure 3. *Representative images of T9-T11 segments demonstrating GFP expression at injury site after T10 contusion SCI followed by intraspinal injections of AAV-1,2,5,9,10,11 serotypes.*

Methods.
Adult female Sprague-Dawley rats (~210g) received contusion injury (150KDyn) at T10 level, as described above. Vectors administration: immediately after injury animals received one of six AAV serotypes expressing

GFP (AAV-gfp), i.e. AAV-1($3.69x10^{13}$), AAV-2($8x10^{12}$), AAV-5($1.9x10^{13}$), AAV-9($1.8x10^{13}$), AAV-rh10($4.16x10^{13}$), AAV-hu11($7.7x10^{12}$) using either an intraspinal, intrathecal or intramuscular injections. n = 3/serotype/administration rout (i.e. n = 9 rats/serotype). Intraspinal injections: 4 injections of 0.5µl each in the left and right ventral horn 1 mm rostral and 1 mm caudal to injury. Intrathecal injections: a total volume of 5µl was slowly injected into CSF through a small hole made in the dura. Intramuscular injections: 20µl of virus was injected into multiple sites in the Femoris and Semitendinosus muscles of the left hind-limb and into the Triceps and Biceps Brachii muscles of left fore-limb. After 10 weeks post-injury and AAV vectors administration spinal cord tissue was collected and cut on cryostat for evaluation of GFP–positive neurons, axons and glial cells in the vicinity of the injury (T9-T11 horizontal sections) and lumbar segments (L1-L5 cross-sections).

Results.

Neurons

Serotypes	Intraspinal	Intramuscular	Intrathecal
AAV-1	+++ $(!!!)_{n=1}$ ++ $(!!!)_{n=2}$	+(!) $_{n=3}$	+(!!) $_{n=1}$ - $_{n=2}$
AAV-2	++++ $(!!!)_{n=2}$ +++(!!!) $_{n=1}$	- $_{n=3}$	++(!!!) $_{n=2}$ +(!!!) $_{n=1}$
AAV-5	++++ $(!!!)_{n=2}$ +++(!!!) $_{n=1}$	- $_{n=3}$	+(!!) $_{n=3}$
AAV-9	+++ $(!!!)_{n=1}$ ++ $(!!!)_{n=2}$	+(!!) $_{n=3}$	+++(!!!) $_{n=1}$ ++ $(!!!)_{n=2}$
AAV-rh10	+++(!!!) $_{n=1}$ ++(!!!) $_{n=2}$	++(!!) $_{n=3}$	++(!!!) $_{n=3}$
AAV-hu11	++ $(!!)_{n=2}$ + $(!!)_{n=1}$	+(!) $_{n=3}$	+(!) $_{n=3}$

Fibers

Serotypes	Intraspinal	Intramuscular	Intrathecal
AAV-1	++++(!!!) $_{n=3}$	+(!) $_{n=3}$	++(!!)$_{n=1}$ - $_{n=2}$
AAV-2	++(!!!) $_{n=3}$	- $_{n=3}$	++(!!!)$_{n=3}$
AAV-5	+++ $(!!!)_{n=3}$	- $_{n=3}$	+(!!) $_{n=3}$
AAV-9	++++(!!!) $_{n=2}$ +++ $(!!!)_{n=1}$	++(!!) $_{n=3}$	++++(!!!) $_{n=1}$ +++(!!!) $_{n=2}$
AAV-rh10	++++(!!!) $_{n=2}$ +++ $(!!!)_{n=1}$	++(!!) $_{n=3}$	**+++(!!!) $_{n=3}$**
AAV-hu11	++ $(!!)_{n=1}$ + $(!!)_{n=2}$	+(!) $_{n=3}$	+(!) $_{n=3}$

Glial cells

Serotypes	Intraspinal	Intramuscular	Intrathecal
AAV-1	+(!!)$_{n=2}$ - $_{n=1}$	+(!)$_{n=1}$ - $_{n=2}$	+(!!) $_{n=1}$ - $_{n=2}$
AAV-2	+(!!)$_{n=3}$	- $_{n=1}$	+(!!)$_{n=1}$ - $_{n=2}$
AAV-5	+++(!!)$_{n=1}$ ++(!!) $_{n=2}$	- $_{n=3}$	+(!!)$_{n=3}$
AAV-9	+(!!) $_{n=3}$	++(!!) $_{n=1}$ +(!!) $_{n=2}$	++(!!) $_{n=1}$ +(!!) $_{n=2}$
AAV-rh10	+++(!!!) $_{n=3}$	+++(!!) $_{n=3}$	**++++(!!!) $_{n=1}$ +++(!!!) $_{n=2}$**
AAV-hu11	+++(!!)$_{n=3}$	++ (!)$_{n=3}$	++(!) $_{n=3}$

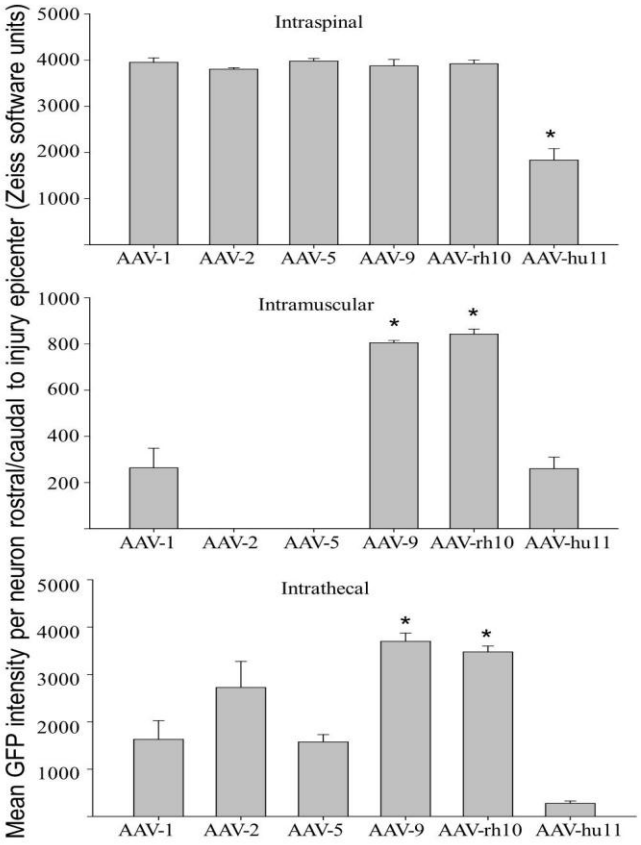

Figure 4. *Scoring number of transduced cells or fibers: - no transduction, + few positive cells/fibers, ++ some transduced cells/fibers, +++ many positive cells/fibers, ++++ robust transduction*
Scoring intensity of transduced cells or fibers: (!) low intensity, (!!) medium intensity, (!!!) high intensity.

Intraspinal injections: transduction efficacy of all six AAV serotypes was comparable (except low signal for AAV-11). Intramuscular injections: AAV9 and AAV10 – markedly stronger gfp signal. Intrathecal administration: AAV9 and AAV10 - markedly stronger gfp signal throughout spinal cord tissue.

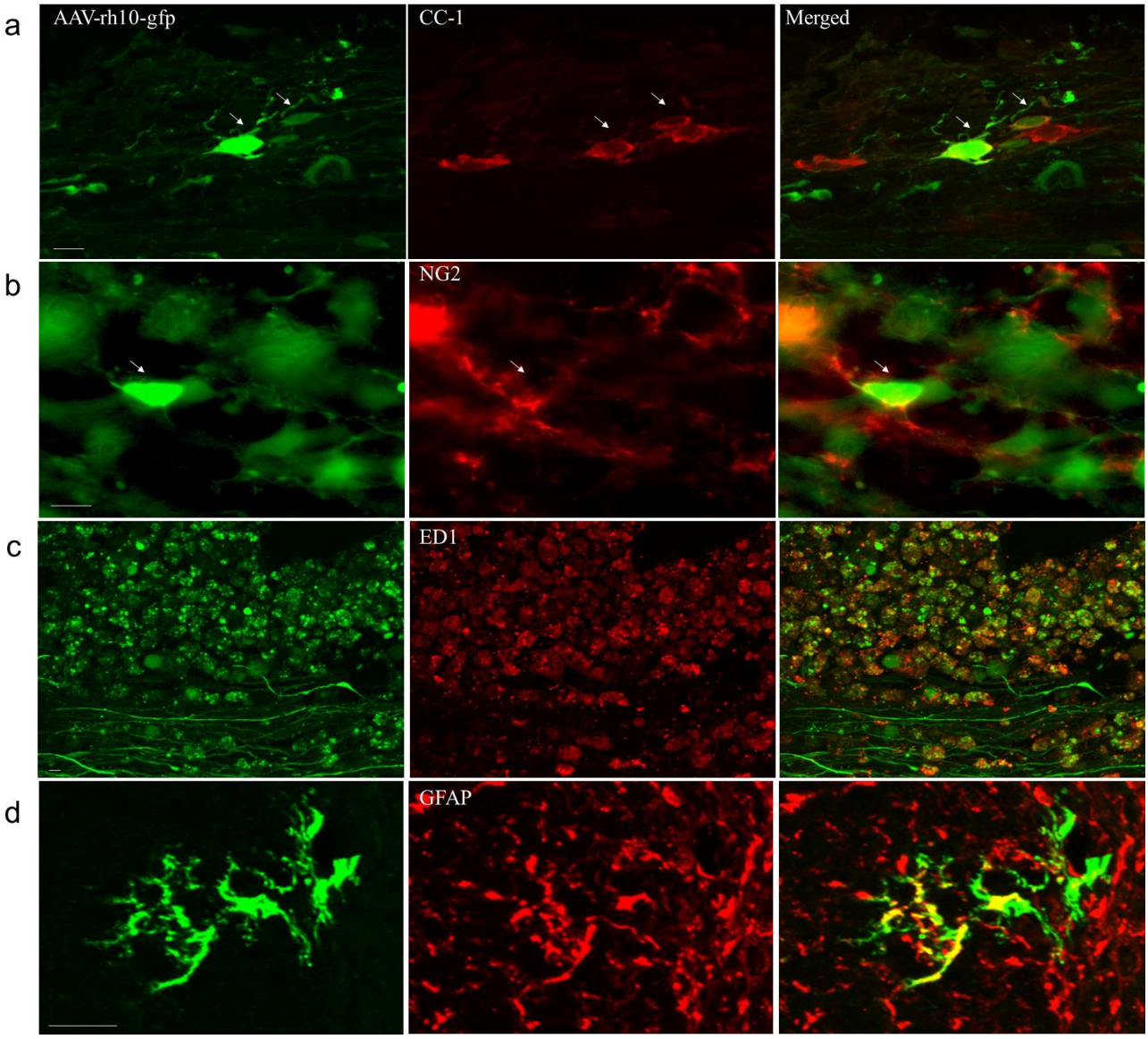

Figure 5. *Transduction of cells by AAV-rh10-gfp following its intrathecal administration immediately after contusion. T9-T11 horizontal sections immunostained with: (a) CC-1 for oligodendrocytes, (b) anti-NG2 for NG2 positive cells/processes, (c) ED1 for microglia/macrophages and (d) anti-GFAP to detect astrocytes. Left column: AAV10-gfp transduced gfp positive cells (green). Middle column: positive cells for each antibody used (red). Right column: merged images. Arrows on selected images indicate double labeled cells. Scale bar - 10 µm.*

Conclusion.

- All AAV serotypes (1,2,5,9,rh10,hu11) showed robust transduction of spinal cord tissue following intraspinal injections.
- Only AAV-9 and AAV-rh10 induced robust transduction of spinal tissue following intrathecal injection, markedly greater compared to other (AAV1,2,5,hu11) serotypes.
- Intrathecal AAV-9 and AAV-rh10 induced comparable transduction in neurons and axons throughout spinal cord tissue.
- Intrathecal AAV10-gfp mediated best transduction of glial cells (microglia, astrocytes, NG2 postive cells and oligodendrocytes) and macrophages in the vicinity of contusion.
- AAV-rh10 would be the optimal serotype for targeting glial cells.

(4) **Examined effects of AAV10-mediated delivery of NG2-Ab in rats after HX and contusion SCI**
During year 3, we have conducted experiments using intraspinal injections of AAV10-NG2Ab combined with AAV10 vector expressing neurotrophin 3 (AAV10-NT3-gfp) in adult rat spinal cord following HX and contusion SCI. We have used a battery of behavioral tests to assess effects of treatment on locomotor function. To evaluate effects of treatments the following tests have been carried out: Open-field locomotion, Irregular Ladder, Narrowing Beam (as described in Arvanian et al., 2009; Schnell et al., 2011) and Catwalk gait analysis (as described in Petrosyan et al., 2013). Prior to injury, animals were trained to cross the Ladder, Beam and Catwalk runways.

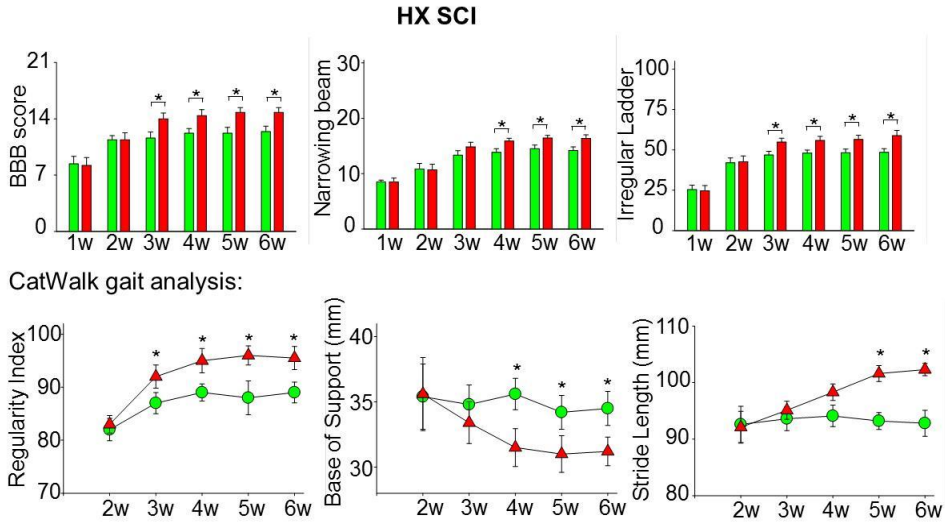

Fig. 6. *Treatment with AAV-NG2Ab plus AAV-NT3 improved locomotor function after HX SCI. Recovery in BBB, two challenging tests (Narrowing beam and Irregular ladder) and CatWalk gait. Data presented as mean±SE*
** P < 0.05.*
n = 6/group

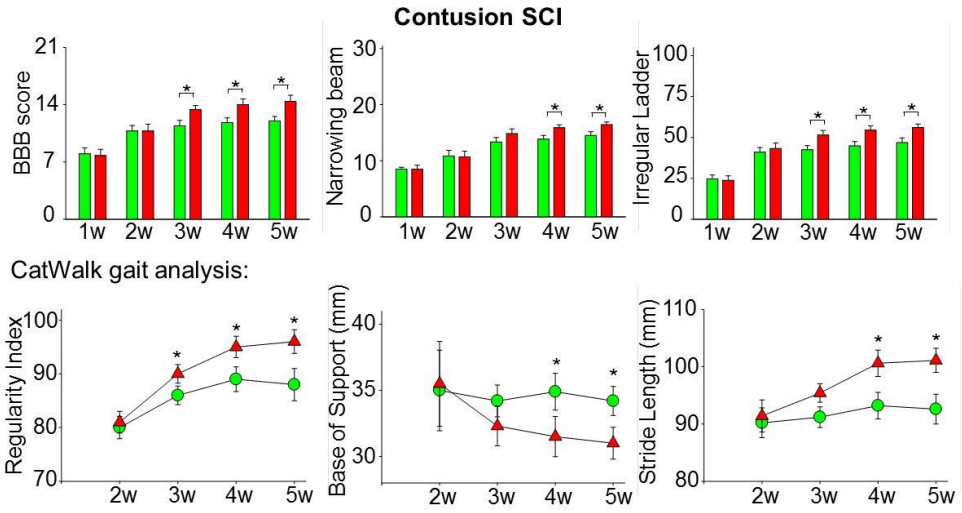

Fig. 7. *Treatment with AAV-NG2Ab plus AAV-NT3 improved locomotor function after contusion SCI. n = 6/group*

BBB. Rats were observed in an open field and BBB testing was carried out by two independent observers for 4 minutes. Joint movements, weight support, paw placement and coordination were evaluated according to the 21-point BBB locomotion scale.

Irregular Ladder. The animals were required to cross a 1-meter long horizontal ladder elevated 30 cm above the ground. A defined stretch of 60 cm was chosen for analysis. To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (1–4 cm spacing). The animals crossed the Ladder Rung Walk twice in the same direction and once in the opposite direction. The number of errors (any kind of foot slip or total miss) was divided by the total number of steps in each crossing, yielding the percentage of missteps.

Narrowing Beam. This paradigm assesses the ability of the rats to balance across a tapered beam 20 cm above the ground. The beam is graded into 30 stretches of the same length, but different width, starting with 5 cm and ending with 1.5 cm width and can be crossed easily by an intact animal. The maximum possible score in this test is 30. The unit at which the first slip of either hindlimb was made was counted and normalized for three runs.

CatWalk. Assessment of locomotor function was completed using the CatWalk device (Noldus Information Technology). Animals crossed the runway where their footprints were captured by a high-speed camcorder. Data from 3 complete uninterrupted runs for each animal were collected and analyzed using CatWalk XT software. Gait parameters, such as Regularity Index and Base of Support, were collected and compared between groups. These parameters have been reported as objective measurements of locomotor performance and coordination after spinal cord injury (Petrosyan et al., 2013).

Results of these experiments presented in Figs. 6, 7. Encouragingly, our results strongly suggest that treatment with AAV10-NG2Ab and AAV10-NT3-gfp (administered together immediately after SCI) improved locomotor function following HX (Fig. 6) and contusion (Fig. 7) SCI.

Thus all 4 specific aims of the project have been successfully accomplished. We apply for 6-moths no-cost extension to complete post-mortem immunochemistry analyses of spinal cords that were removed from animals after completion of behavioral testing described in Figs 6, 7. These analyses will address following questions: (1) what types of cells (i.e. neurons, oligodendrocytes, microglia and macrophages) have been transduced in damaged spinal cord treated with AAV10-NG2-Ab; (2) if administration of AAV-NG2Ab following SCI may generate additional macrophage/microglia cellular inflammatory response.

KEY RESEARCH ACCOMPLISHMENTS:

- We have conducted single-cell electrophysiological recording following chronic contusion SCI in adult rats. We have demonstrated that after thoracic chronic contusion the disrupted dorsal corticospinal tract axons spontaneously form new synaptic contacts with individual motoneurons, extending around the contusion cavity, through spared ventrolateral white matter. These detour synaptic connections are very weak and strengthening these connections in order to improve function may be a target for therapeutic interventions following SCI.
- The recombinant single chain (scFv) for NG2-Ab has been created as planned, AAV-mediated expression of NG2-Ab has been confirmed (Levine lab), plasmid has been send to PENN vector core.
- Optimal AAV vector serotype for NG2-Ab delivery following SCI has been determined. We have compared expression pattern of six AAV serotypes and found that AAV-10 serotype induced best transduction of spinal cord tissue following contusion SCI.
- Based on these experiments, novel AAV-10 vector-based construct expressing NG2-antibody (AAV10-NG2-Ab) has been created (PENN vector core) as planned.
- Effects of prolonged delivery of NG2-Ab using AAV10-NG2-Ab construct on recovery of motor function following SCI have been examined. We found that AAV10-mediated delivery of NG2-Ab and neurotrophin NT3 expressing units significantly improved locomotor function after hemisection and contusion SCI.

CONCLUSIONS.

1. We have completed experiments described in all four Specific Aims of the Project. Our experiments related to the chronic delivery of NG2-Ab via osmotic mini-pump revealed that chronic delivery of NG2-Ab via intrathecal catheter and osmotic mini-pump induce partial recovery of synaptic transmission, improved anatomical plasticity and facilitated recovery of locomotor function after SCI. Although this is a great proof of principle, some recent clinical studies (Novartis clinical trials) showed that similar delivery of therapeutic agents using catheter implantation may have potential problems, such as inflammations and clogging of the tip of the catheter. Thus gene therapy using AAV viral vector-mediated delivery of NG2-Ab expressing units may have a better translational potential.

2. During Year3 we have examined transmission in damaged spinal cord following contusion SCI in adult rats, which is more realistic model of spinal injuries.

3. Moreover, during year 3 we have successfully created the recombinant single chain (scFv) antibody (patent is pending). The plasmid has been transferred to PENN vector core, where the AAV10 viral vector encoding the anti-NG2 scFv has been constructed. The choice of AAV-10 serotype for this new construct was based on our experiments that revealed that among several AAV-gfp serotypes that we tested (AAV1,2,5,9,10,11), AAV10-gfp induced best transduction of spinal cord tissue following contusion SCI.

4. We further examined effects of AAV10-mediated delivery of NG2-Ab combined with neurotrophin NT3 in rats that received either hemisection or contusion SCI. We found that rats that received SCI and AAV10-

NG2-Ab plus NT-3 treatment exhibited significantly better recovery of locomotor function compared with control group that received identical SCI and control AAV10-gfp injections.

5. Our study proved that AAV10-NG2-Ab construct that we created may be a novel, effective and clinically relevant treatment to facilitate recovery after SCI.

6. During 2012-2013 period we published four papers. Some recent results described above have been summarized in three published 2013SFN abstracts and three other papers are being prepared for publication.

7. During pending 6-months no-cost extension of the Project we will complete immunochemistry analyses of the spinal cords (from experiment described in part 4 of this Conclusion), in order to detect expression pattern of neurons and glial cells in spinal cords that received SCI and AAV10-NG2-Ab treatment.

REPORTABLE OUTCOMES and REFERENCES:

Manuscripts Published in Peer- Reviewed Magazines during 2012-2013:

1. Hunanyan, A.S., Petrosyan, H.A., Alessi, V. & Arvanian, V.L. Repetitive spinal electromagnetic stimulation opens a window of synaptic plasticity in damaged spinal cord: role of NMDA receptors. Journal of Neurophysiology, 107, 3027-3039, 2012.
2. Arvanian V. Role of Neurotrophins in Spinal Plasticity and Locomotion. Current Pharmaceutical Design. Review. 19:4509-16, 2013.
3. Petrosyan, H.A., Hunanyan, A.S., Alessi, V., Schnell, L., Levine, J. & Arvanian, V.L. Neutralization of inhibitory molecule NG2 improves synaptic transmission, retrograde transport and locomotor function after spinal cord injury in adult rats. Journal of Neuroscience. 33, 4032-43, 2013.
4. Hunanyan, A.S., Petrosyan, H.A., Alessi, V., Arvanian, V.L. Combination of Chondroitinase ABC and AAV-NT3 promotes neural plasticity at descending spinal pathways following thoracic contusion in rats. J Neurophysiol. 2013 (Jul 17, PMID: 23864374).

2013 SFN Abstracts:

H. A. Petrosyan, A. S. Hunanyan, V. Alessi, S. Sandler, J. M. Levine, V. L. Arvanian (2013) AAV10 based gene therapy to neutralize inhibitory action of NG2 and deliver neurotrophin NT-3, improves function following thoracic contusion and hemisection lesion spinal cord injuries. Society for Neuroscience, San Diego CA, Program No.168.03/PP10.

H. A. Petrosyan, A. S. Hunanyan, V. Alessi, S. Sandler, J.M. Levine, V. L. Arvanian (2013) Spinal electro-magnetic stimulation improves synaptic plasticity in contusive spinal cord in an activity-dependent manner. Society for Neuroscience, San Diego CA, Program No.168.10/PP17.

A. S. Hunanyan, H. A. Petrosyan, V. Alessi, V. L. Arvanian (2013) Transmission from the motor cortex to spinal cord neurons and limb muscles after unilateral cortical lesion (TBI) in adult rats. Society for Neuroscience, San Diego CA, Program No. 442.03/AA6.